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Franco Matías Escobar [a], María Carola Sabini [a], Silvia Matilde Zanon [a], Carlos Eugenio Tonn [b] & Liliana Inés Sabini [a]

[a] Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas Físico, Químicas y Naturales de la Universidad Nacional de Río Cuarto, Río Cuarto, Córdoba, Argentina

[b] INTEQUI-CONICET, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, San Luis, Argentina

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SHORT COMMUNICATION

Antiviral effect and mode of action of methanolic extract of *Verbascum thapsus* L. on pseudorabies virus (strain RC/79)

Franco Matías Escobar[a], María Carola Sabini[a], Silvia Matilde Zanon[a], Carlos Eugenio Tonn[b] and Liliana Inés Sabini[a]*

[a]*Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas Físico, Químicas y Naturales de la Universidad Nacional de Río Cuarto, Río Cuarto, Córdoba, Argentina;* [b]*INTEQUI-CONICET, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, San Luis, Argentina*

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The methanolic extract of *Verbascum thapsus* was evaluated for its antiviral activity against the pseudorabies virus strain RC/79 (PrV), and also for its cytotoxic activity on Vero cells. The extract showed CC_{50} values of $1100\,\mu g\,mL^{-1}$ and $1426\,\mu g\,mL^{-1}$ by NRU and MTT assays, respectively. The 50% inhibitory concentration of the extract for PrV plaque formation was determined at $35\,\mu g\,mL^{-1}$, and selectivity indices were 31.4 (NRU) and 40.7 (MTT). When cells were pre-treated with the extract prior to virus infection, the inhibition in plaque formation was 70%. PrV was highly inhibited when it was incubated with plant extract or when the extract was added during the adsorption phase (99%). However, no inhibitory effect was observed when the extract was added to the cells after the adsorption period. Thus, these results suggest that the methanolic extract of *Verbascum thapsus* may contain bioactive compound(s) that affect PrV mostly in the adsorption phase.

Keywords: *Verbascum thapsus* L.; antiviral activity; pseudorabies virus (strain RC/79); methanolic extract

1. Introduction

Pseudorabies virus (PrV; suid herpesvirus 1 (SHV-1), according to the international nomenclature suid) is the aetiological agent of Aujeszky's disease in pigs (Mettenleiter, 2000). It has been classified as a member of the genus *Varicellovirus* within the family Herpesviridae (Roizman & Pellett, 2001). This taxon also includes human herpes simplex virus type 1 and 2.

In the search of effective agents for the treatment of herpesvirus, nucleosides analogues have been heavily investigated (Darby, 1994) including acyclovir and derivatives, ganciclovir, foscarnet and cidofovir, all of which inhibit herpesvirus DNA polymerase. Some of these antiviral agents, e.g., ganciclovir and foscarnet may produce toxic side-effects. In addition, the emergence of resistant virus strains to

*Corresponding author. Email: lsabini@exa.unrc.edu.ar

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commonly used antiherpetic drugs is an increasing problem, particularly in immune-compromised patients (Cassady & Whitley, 1997; Reusser, 1996). Therefore, there is a great interest in the search for new anti-herpesvirus drugs.

In recent years, interest in alternative therapies and the therapeutic use of natural products has been growing, especially those derived from plants (Rates, 2001).

Verbascum thapsus L. is a species of Scrophulariaceae family with worldwide distribution, growing in uncultivated land, slopes and dry meadows. It is native to Europe and Asia (Semenza, Young, & Evans, 1978), and has been introduced throughout the temperate world, including South America. *Verbascum thapsu*s is commonly known as mullein, candelaria, and gordolobo. In Argentina 'ambay' is the usual name (E.M. Petenatti, M.E. Petenatti, & Del Vitto, 1999).

Traditionally *V. thapsus* has been used to cure headaches, fevers, cramps, burns and other ailments (J.M. Baskin & C.C. Baskin, 1981; Tyler, 1993). Mullein methanolic extract previously showed antibacterial and antifungal activities and partially inhibited the cytopathic effects of bovine herpesvirus type 1 (McCutcheon, Ellis, Hancock, & Towers, 1992, 1994; McCutcheon et al., 1995). In addition, the ethanolic extract of this plant showed antiviral activity on suid herpesvirus 1 (Zanon, Ceriatti, Rovera, Sabini, & Ramos, 1999).

Studies on the constituents of methanolic extract of *V. thapsus* reported the presence of iridoid glycosides laterioside, harpagoside, ajugol and picroside IV; phenylethyl glycoside verbascoside; iridoids (+)-genipin, α-gardiol and β-gardiol. Sesquiterpenes buddlindeterpene A and buddlindeterpene B, and diterpene buddlindeterpene C have also been identified. The bisflavonoid amentoflavone has been reported from the whole plant (Hussain et al., 2009; Warashina, Miyase, & Ueno, 1991).

Therefore, the main aim of this study was to determine effective inhibitory concentration and the possible mode of action of *Verbascum thapsus* L. methanolic extract at different stages in the PrV infection cycle on Vero cells. Previously it was necessary to evaluate cytotoxic property of the extract, due to the obligate parasitism of the virus.

2. Results and discussion

2.1. *Cytotoxic activity of methanolic extract of* Verbascum thapsus *L. on Vero cells*

Cytotoxic effect of methanolic extract of *V. thapsus* on Vero cells was evaluated using MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma–Aldrich method (Mosmann, 1983) and Neutral Red Uptake assay (NRU) in a modified form as described by Borenfreund and Puerner (1985). In addition, the maximum non cytotoxic concentration (MNCC) was determined.

Results showed that methanolic extract produced a dose-dependent inhibition on the Vero cells growth (Figure 1(a)). The corresponding 50% cytotoxic concentration (CC_{50}) found was $1100\,\mu g\,mL^{-1}$ and $1426\,\mu g\,mL^{-1}$ by NRU and MTT assays, respectively. The MNCC value was $850\,\mu g\,mL^{-1}$. At this concentration, the survival rate was 64% and 99%, in the NRU and MTT assays, respectively. This difference could be due to the fact that the plasma membrane (NRU assays), which is the first site exposed to the extract, is attacked more easily than the mitochondrial one (MTT assays), as suggested by Bouaziz, Abid-Essefi, Bouslimi, El Golli, and Bacha (2006).

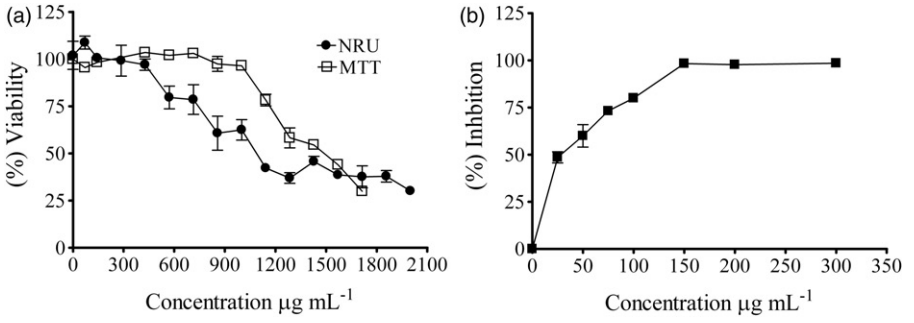

Figure 1. (a) Percentage of viability of cultured Vero cells, incubated 48 h in the presence of methanolic extract of *Verbascum thapsus* L. at different concentrations determined by (•) NRU and (□) MTT assays. CC_{50} are 1100 and 1426 $\mu g\,mL^{-1}$, by NRU and MTT assays, respectively. (b) Effects of methanolic extract of *Verbascum thapsus* L. on plaque formation by pseudorabies virus. Methanolic extract of *V. thapsus* shows 50% inhibition of plaque formation at 35 $\mu g\,mL^{-1}$. Each value represents the mean ± standard error mean.

The methanolic extract at 300 $\mu g\,mL^{-1}$ did not affect cell growth (data not shown), showing a cell survival rate close to 100% for both assays. Therefore, only concentrations $\leq$300 $\mu g\,mL^{-1}$ were selected for later studies.

2.2. *Antiviral activity*

The 50% inhibitory concentration (IC_{50}) value of the methanolic extract on PrV infection was investigated by plaques reduction. The extract exhibited a clear dose-response in PrV infection, Figure 1(b). The percentage inhibition in plaque reduction assay increased from 40.5 to 99.4% as the concentration of methanolic extract rose from 25 to 300 $\mu g\,mL^{-1}$. The 50% of pseudorabies virus production was inhibited by 35 $\mu g\,mL^{-1}$(IC_{50}) of methanolic extract. The selectivity index (SI) was determined by the ratio of CC_{50} to IC_{50}. The values for the methanolic extract were 31.4 (NRU) and 40.7 (MTT). These results and particularly the difference between their CC_{50} and MNCC values, suggest a promising future for this extract as antiviral product.

2.2.1. *Some evidences of the mode of antiviral action*

Pseudorabies virus replication is characterised by a complex sequence of different steps which offers antiviral agents the opportunity to interfere. In order to identify the antiviral target site, methanolic extract was added at non-cytotoxic concentration during different infection stages (Table 1). The percentage reduction was calculated relative to the amount of virus produced in the absence of the extract. Treatment of virus with the methanolic extract prior to infection caused a significant reduction of infectivity of PrV, 90% and 98% at 4°C or 37°C, respectively, compared to controls. This result suggests that the extract interacts with structures of the viral envelope which is necessary for adsorption or entry into host cells.

On the other hand, pre-treatment of host cells with the extract prior to virus infection exhibited a significant plaque reduction of 70% for PrV. This data indicates that the extract not only interacts with the envelope of herpesvirus but also with the

Table 1. Antiviral effect of the methanolic extract of *Verbascum thapsus* L. on pseudorabies virus by incubation at different infection steps.

Adsorption	Penetration	Pretreatment cells	Pretreatment virus 4°C	37°C	Intracellular replication
99	10	70	98	90	10

Note: Results are given in plaque forming units expressed as per cent of virus control.

surface of the host cells. When the methanolic extract was added during the adsorption period, viral production was significantly reduced to 99%. However, when the extract was added to the cells after virus adsorption, the number of plaques was not considerably reduced. These results suggest that pseudorabies virus is inactivated before adsorption and also during adsorption to cell surface but not after penetration into host cells. The virucidal activity indicates that methanolic extract of *V. thapsus* might be further explored as a virucidal agent.

3. Conclusions

The methanolic extract of *V. thapsus* selectively inhibits the replication of pseudorabies virus, probably before adsorption or during adsorption. Further analysis and purification steps are required in order to identify the active principles and clarify the chemical nature of the potential anti-PrV molecules.

Supplementary material

Experimental details relating to this article are available online.

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